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On-channel base stacking in microchip capillary gel electrophoresis for high-sensitivity DNA fragment analysis

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Abstract

We evaluated a novel strategy for high-sensitivity DNA fragment analysis in a conventional glass double-T microfluidic chip. The microchip allows for a DNA on-channel concentration based on base stacking (BS) with a microchip capillary gel electrophoretic (MCGE) separation step in a poly(vinylpyrrolidone) (PVP) sieving matrix. Depending if low conductivity caused a neutralization reaction between the hydroxide ions and the run buffer component $Tris^+$, the stacking of DNA fragments were processed in the microchip. Compared to a conventional MCGE separation with a normal electrokinetic injection, the peak heights of 50–2650-base pair (bp) DNA fragments on the MCGE-BS separation were increased 3.9–8.0-fold. When we applied the MCGE-BS method to the analysis of a clinical sample of bovine *theileria* after PCR reaction, the peak height intensity of the amplified 816-bp DNA fragment from the 18S rRNA of *T. buffeli* was enhanced 7.0-fold compared to that of the normal injection method.

Keywords: Chip technology; DNA; Concentration on-channel; Base stacking; Stacking

1. Introduction

Microchip capillary electrophoresis (MCE) is becoming a powerful and effective analytical technique for DNA analysis due to its analytical throughput, speed, small reagent volume, automation, miniaturization, and high resolution [1–15]. The most significant advantage of MCE in DNA fragment analysis is its high speed compared to conventional slab gel electrophoresis. However, although MCE has advantages over slab gel electrophoresis, a major limitation of MCE analysis is the small sample volume, which causes a low concentration sensitivity. Developing an on-line pre-concentration technique that can be used for conventional commercial MCE apparatuses is generally desirable [11–18], because changing the microchip design to enhance the injection amount and the detection window length can cause another problem such as band broadening [19,20]. There are a few approaches to this matter that describe pre-concentrated DNA samples in the microchip channel prior to separation [11–15]. Solid-phase extraction (SPE) used a microchip with C_{18}-coated channels and octadecylsilane-coated channel [21,22]. The SPE method increased detection sensitivity 80- and 500-fold. However, it is not easy to apply this method to a microchip, as it involves complex coating or packing the microchip channel that has been sealed, usually irreversibly. Another method, isotachophoresis (ITP) uses a binary buffer system to make the sample constituents between a leading and a terminating buffer. Among the methods, ITP is becoming an important technique for DNA pre-concentration on microchips [14,15] since Bodor et al. [23] first reported microfluidic devices that utilized integrated sample pre-concentration by ITP, to detect food additives. The ITP method has the advantage of improving the detection sensitivity without a loss in peak resolution. However, although ITP could be a powerful DNA sample concentration technique, it is not a simple method. This is because ITP needs another buffer system, such as leading electrolyte and terminating electrolyte, as

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well as a long sample injection time. A modification of chip design is also necessary to apply the ITP technique to MCE [15].

In capillary electrophoresis (CE), the base stacking (BS) method enables direct injection of unpurified products of dye-primer sequencing reactions into capillaries without any pre-treatments. On-column concentration of DNA fragments in CE is achieved simply by an electrokinetic injection of hydroxide (OH^-) ions. A neutralization reaction between the OH^- ions and the run buffer component $Tris^+$ results in a zone of lower conductivity, within which field focusing occurs. The BS technique was first proposed by Xiong et al. and was applied to the sample concentration method for capillary-based DNA sequencing [24]. Zhang et al. also applied the BS technique for the high-throughput polymerase chain reaction (PCR) analysis of clinical samples by CE [25]. Unlike other on-line pre-concentration techniques, the BS method is very simple, since the introduction step of OH^- ions is needed only before the injection of DNA sample. However, there are no reports regarding on-line DNA pre-concentration by the BS technique in MCE, in spite of its simplicity.

In this study, we developed a microchip capillary gel electrophoretic (MCGE) separation method with a BS (MCGE-BS) as an on-line pre-concentration technique, which uses just one run buffer system, to enhance DNA fragment sensitivity and resolution efficiency without having to modify the chip design. Since the BS occurs simultaneously with the electrokinetic injection of OH^- and DNA sample, the stacking is initiated at the low-conductivity zone of the microchip channel, which results in an increased DNA sample concentration. Base stacking of DNA fragments, in a glass microchip, with an applied electric field that uses a conventional double-T microchip, is demonstrated for high-sensitivity PCR DNA fragment analysis without any chip modification.

2. Experimental

2.1. Chemical and reagents

A 1× TE buffer (50 mM Tris–HCl, 2 mM EDTA, pH 8.0) was prepared by dissolving in proportion Trizma-base, Trizma-hydrochloride, and EDTA disodium (all from Sigma, St. Louis, MO, USA) in deionized water. The microchip channel sieving matrix was made by dissolving 2% (w/v) of 1 000 000 M_r poly(vinylpyrrolidone) (PVP) from Polyscience (Warrington, England) into the 1× TE buffer with 0.5 μg/ml ethidium bromide (EtBr) (St. Louis, MO, USA). A 50-bp DNA ladder was purchased from Invitrogen (Carlsbad, USA). For the whole blood PCR of bovine *theileria*, 10× PCR buffer, 25 mM $MgCl_2$, formamide, EtBr and 2.5 mM dNTP mix were purchased from Promega (Madison, WI, USA). The *Taq* DNA polymerase (5 U/μl) was obtained from Super-Bio (Suwon, Korea). The forward (5′-AAA CTG CGA ATG GCT CAT-3′) and reverse (5′-ACA TCC TTG GCA AAT GCT-3′) primers synthesized by GenoTech (Daejeon, South Korea) were used for the amplification of an 816-bp DNA fragment from 18S rRNA of *T. buffeli* (buffeli/orientalis/sergenti).

2.2. PCR sample preparation

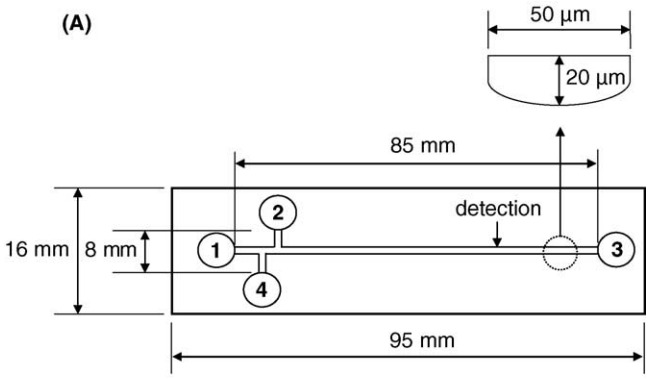
In bovine *theileria* PCR, the amplified fragment 816-bp DNA from the 18S rRNA of *T. buffeli* was obtained directly from 200 nl of a whole blood sample. The reaction was performed in a thermal cycler (Perkin-Elmer model 2400, USA) using the same procedure, described by Kang and co-workers [26,27], at the following temperatures: 10 min incubation at 80 °C; 40 cycles of denaturing at 80 °C for 30 s, annealing at 40 °C for 60 s, and extension at 60 °C for 60 s; followed by a 7 min hold at 60 °C. The 10 μl PCR reaction mixture had the following final composition: 3 mM $MgCl_2$, 1 μl of 10× PCR buffer, 0.25 mM of dNTP, 0.4 μM of each forward and reverse primer, 16% formamide, 2 U of *Taq* DNA polymerase and 200 nl of whole blood (or purified DNA). Finally, each amplified product was introduced into the MCE or CE system.

2.3. Capillary electrophoresis for conductivity measurement

The experimental CE setup for a conductivity measurement was similar to that described in [28]. To measure the conductivity of the fluid in the capillary, a 1 kΩ resistor was inserted between the waste reservoir electrode and the ground. A Spellman 1000R high-voltage power supply (Spellman High Voltage, Hauppauge, USA) was used to drive electrophoresis. A 60 cm (30 cm effective length) × 50 μm i.d. bared fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) were used as a separation capillary. The run buffer was composed of 1× TE buffer (pH 8.0) with 0.5 μg/ml EtBr. The capillary sieving matrix was made by dissolving 4% (w/v) of 1 000 000 M_r PVP into the 1× TE buffer with 0.5 μg/ml EtBr. After the sample was injected electrokinetically at 50 V/cm for 30 s, 0.1 M NaOH was injected at 150 V/cm for 60 s for stacking. Finally, 150 V/cm was performed for the separation. After each run, the capillary was reconditioned, prior to the next analysis, by rinsing it for 10 min in the following sequence: water, 0.1 M NaOH, water, and the run buffer. The data was recorded as a function of time during the CE, and saved in an IBM-compatible computer (1.70 GHz Pentium IV) at 10 Hz. Data treatment and analysis were performed using an Autochro data system (Young Lin Instrument Co., Anyang, Korea).

2.4. Microchip capillary electrophoresis

MCE was performed on a DBCE-100 Microchip CE system (Digital Bio Technology, South Korea) equipped with



(B)

Reservoir	1	2	3	4
OH^- injection voltage (kV)	0.48	0.08	0.08	0.00
Sample injection voltage (kV)	0.24	0.00	0.24	0.48
Separation voltage (kV)	0.00	0.17	1.20	0.17

Fig. 1. (A) Schematic diagram of microfluidic chip. (B) The applied injection times of OH^- and DNA sample and the separation voltage for base stacking.

a diode-pumped solid-state laser (exciting at 532 nm and collecting fluorescence at 605 nm; Power Technology, Little Rock, AZ, USA), and a high-voltage device (DBHV-100, Digital Bio Technology). The microfluidic chip was a standard microfluidic chip (MC-BF4-TT100, Micralyne, USA). The chip channel was 50 μm wide and 20 μm deep. The reservoirs were 2.0 mm in diameter and 1 mm deep. A double-T injector with a 100 μm offset was selected as a conventional microfluidic chip. The injection channel length (from reservoir 2 to reservoir 4 in Fig. 1A) was 8.0 mm. The separation channel (from reservoir 1 to reservoir 3 in Fig. 1A) was 85 mm long and detection was performed at 50 mm from the injection-T. All the reservoir positions are shown in Fig. 1A. The run buffer was 1× TE buffer with 0.5 μg/ml EtBr. The microchip channel sieving matrix was made by dissolving 2% (w/v) of 1 000 000 M_r PVP into the 1× TE buffer with 0.5 μg/ml EtBr, shaking for 2 min and letting it stand for 1 h to remove any bubbles. The sieving matrix was hydrodynamically filled by subjecting the vacuum of 8.67×10^4 Pa (EYELA A-3S vacuum aspirator, Tokyo Rikakikai, Japan) to the MCE reservoir 3 for 3 min. The sample was pipetted into sample inlet reservoir 2 of the microchip. The normal sample injection by conventional electrokinetic injection, was accomplished in the injection-T region by applying a potential of 480 V at the sample outlet reservoir 4, followed by grounding the sample inlet reservoir 2 for 60 s (Fig. 1B). Subsequently, separation was achieved by applying potentials of 0, 170, 1200 and 170 V at the buffer inlet (1), sample inlet (2), buffer waste (3) and NaOH inlet (and/or sample outlet) (4), respectively. After each run, the microchip channel was rinsed in the following sequence: water, 0.1 M NaOH, water, and run buffer for 5 min each. The peak

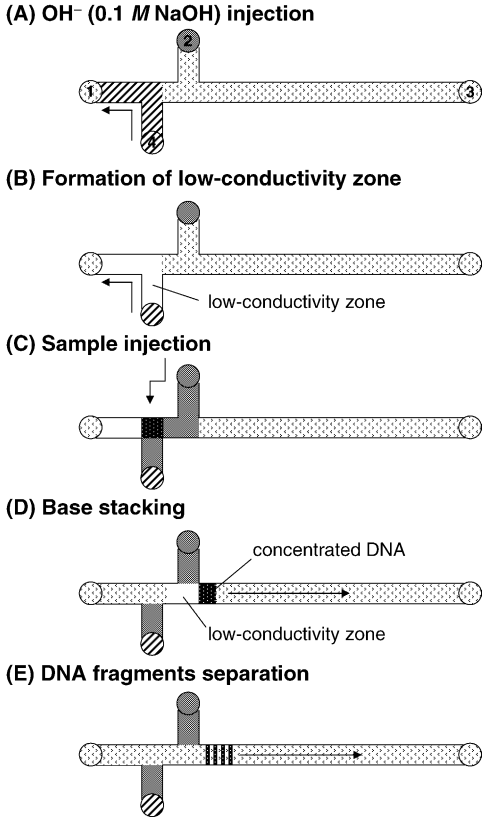


Fig. 2. Schematic mechanism of base stacking with MCGE separation using a conventional microchip. The channel length, width, and depth of the microchip were 85 mm, 50 and 20 μm, respectively. Effective length was 50 mm. Reservoirs 1–4 were the buffer inlet, the sample inlet, the buffer waste reservoir and the OH^- inlet (and/or the sample outlet), respectively. Arrows indicate the fluidic direction. Other indicators are as follows: run buffer (⁙), sample (▒), NaOH (▨), low conductivity (□) and concentrated sample (■).

height and the peak areas of DNA fragments were calculated by OriginPro 7.5 software (OriginLab, Northampton, MA, USA).

2.5. Base stacking in microchip capillary electrophoresis

All DNA samples and reagents for BS were used in the conventional electrokinetic injection method. The BS procedure was carried out in the following three steps: (1) Inject OH^- (0.1 M NaOH) into the channel (1× TE buffer) using the electrokinetic injection method, which applies 533.3 V/cm at the buffer inlet reservoir 1 and grounding the 0.1 M NaOH inlet reservoir 4 for 20–40 s (Fig. 2A). (2) Inject the DNA sample into the channel by applying 600 V/cm at the NaOH inlet reservoir 4 and grounding the sample inlet reservoir 2 for 60 s (Fig. 2C). (3) Begin BS and separation (Fig. 2D and E) by applying 117.6–152.9 V/cm at the buffer outlet reservoir 3 and grounding the buffer inlet reservoir 1. Migration time was measured from this moment for all experiments.

3. Results and discussion

3.1. Base stacking for DNA fragments

Base stacking of DNA fragments in capillary can be caused by changing the conductivity [24,25]. The OH^- ions neutralize the $Tris^+$ ions to generate a low-conductivity zone. The conductivity in the capillary can be described in terms of the following:

$$L = \frac{1}{R} = \frac{1}{P/I} = \frac{I}{P}$$

where L is conductivity (Ω^{-1}), R is resistance (Ω), P is potential (V), and I is current (A). The conductivity is proportional to the current at a constant voltage. To confirm the formation of a low-conductivity zone, we measured the current during the DNA fragments separation, after the injection of OH^- ions in the CGE conditions as follows: run buffer, 1× TE buffer (pH 8.0) with 0.5 μg/ml EtBr; sieving gel matrix, dissolving 4% (w/v) of 1 000 000 M_r PVP with the 1× TE buffer; applied electric field, 150 V/cm. During the OH^- (0.1 M NaOH) injection into the capillary filled with $Tris^+$, the current decreased. The current gap (~3 μA) at the initial separation step of the CGE-BS proves the formation of the low-conductivity zone in the mixing zone of $Tris^+$ and OH^- in capillary (dotted circle in Fig. 3B). Since OH^- ions are immediately neutralized by the run buffer $Tris^+$ ions when they are injected [24], the maximum current gap appeared at the initial separation step. After a hydroxide injection, base stacking was complete. Separation of DNA fragments was initiated, the low-conductivity zone was dissipated, and the run buffer in the capillary was restored to a homogeneous state. Therefore, the current gradually returned to its original value in a smooth pattern, due to ion migration into the low-conductivity zone until the DNA fragment separation was complete (Fig. 3). Thus, it was determined that a low-conductivity zone would generate much faster than the other zone.

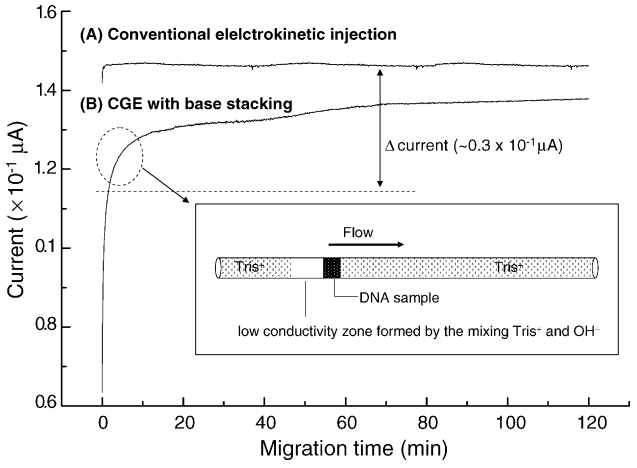


Fig. 3. Comparison of the current at (A) the conventional CGE separation and (B) the CGE separation with base stacking. The bare fused-silica capillary 60 cm (30 cm effective length) × 50 μm i.d. × 365 μm o.d. All DNA sample concentration is 0.5 μg/ml. (A) Conventional electrokinetic injection: DNA sample injection electric field, 50 V/cm for 30 s; OH^- (0.1 M NaOH) injection electric field, 150 V/cm for 60 s; separation electric field, 150 V/cm. (B) Base stacking: sample injection electric field, 50 V/cm for 30 s; separation electric field, 150 V/cm. Box indicates the schematics of the base stacking in CGE. Other indicators are the same as in Fig. 2.

The BS technique cannot use free-solution CE under standard conditions [24], which means that the BS method cannot be used in a free-solution glass microchip. Alternatively BS can be performed using a cathodic injection end, and employing certain self-coating polymer-containing separation buffers that suppress electroosomotic flow (EOF) [29]. We chose PVP as a self-coating replaceable-polymer network. The PVP was also employed to act as a separation matrix for the MCGE separation of DNA fragments. The stacking in MCGE was similar to the sample enhancement in CGE. There was no need for complicated steps, or equipment, and it has better adaptability. The mechanism of BS with MCGE separation that uses a conventional, commercial, double-T microchip is illustrated in Fig. 2. First, OH^- (0.1 M NaOH) ions are injected into the microchannel (Fig. 2A). As soon as the OH^- ions are introduced into the microchip channel, a neutralization reaction between OH^- ions and $Tris^+$ buffer ions occurs, which begins to form a lower-conductivity zone (white regions in Fig. 2B). After injecting the DNA sample (Fig. 2C), stacking of DNA fragments in the microchip channel begins in the low-conductivity zone. Within the zone of high electric field, DNA fragments move at higher velocities than in the untitrated buffer zone. The fast DNA fragments are concentrated at the corner of the neutralized zone (Fig. 2D). Separation starts, the low-conductivity zone dissipates, and MCGE separation proceeds in a PVP sieving matrix (Fig. 2E). All of these steps proceed continuously without stopping.

Baseline separation of DNA fragments is essential for accurate quantitative analysis in MCGE. The peak height and the peak area of DNA fragments increased as the injection time of OH^- (0.1 M NaOH) increased (Fig. 4). When OH^- ions were injected into the microchip channel below 20 s, peaks of DNA fragments were broad because the stacking procedure was insufficient and only small proportional was concentrated (Fig. 4C). As the injection time increased and exceeded 20 s, there was no signal enhancement as peak height, only reduced resolution. This is due to the fact that the over-made neutralization zone reduced the suppressed-EOF and the DNA molecules adsorbed on the surface of microchip. The effective length of the microchip was also not enough to separate all the DNA fragments through baseline separation [24]. In general, the amount of on-line sample stacking (or concentration) in CE is proportional to the electrophoresis-to-sample buffer concentration ratio [30]. Theoretically, a sample prepared in water should give the highest degree of stacking. However, since electroosmosis occurs much more rapidly in the diluted sample than in the electrophoresis buffer, the mismatch in the EOF rate causes a laminar flow inside the capillary, which reduces the sharpness of the stacking process [30,31]. In MCGE-BS, the lower conductivity caused

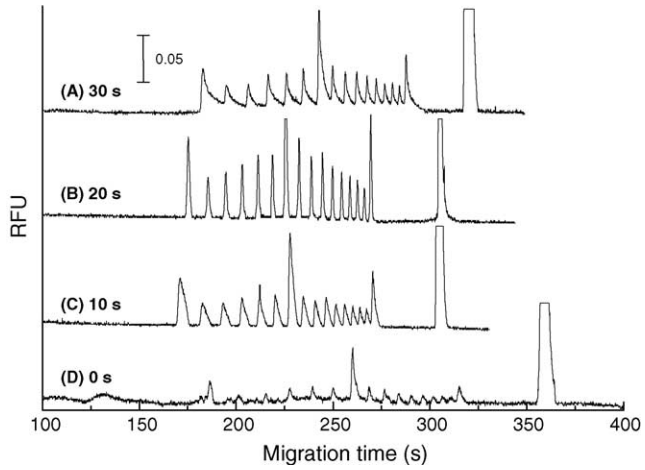


Fig. 4. Comparison of OH^- (0.1 M NaOH) injection time for MCGE separation with BS. A 0.1 M NaOH injection at an applied electric field of 533.3 V/cm for (A) 30 s, (B) 20 s, (C) 10 s and (D) 0 s. Other MCGE conditions: run buffer, 1× TE with 0.5 μg/ml EtBr (pH 8.0 with Trizma-base); sieving matrix, 2% PVP (M_r 1 000 000) in the run buffer; microfluidic glass chip, 85 mm total length × 50 μm width × 20 μm depth, 100 μm double-T injector; 50 mm effective length; sample, 0.5 μg/ml of 50-bp DNA ladder; sample injection with electric field of 600.0 V/cm for 60 s; separation electric field, 141.2 V/cm. RFU: relative fluorescence unit.

by the over-made neutralization zone between the OH^- ions and $Tris^+$ ions affected the suppressed-EOF in the microchannel, and caused the asymmetric peak-broadening during the MCGE separation step in PVP sieving matrix (Fig. 4A). Consequently, the injection time of 20 s was selected as the optimum injection time for OH^- ions at the 100 μm offset, double-T microchip and channel length of 85 mm, under the applied electric field of 141.2 V/cm for MCGE-BS separation (Fig. 4B).

The electric field also affected the resolution and migration time after BS (Fig. 5). When the BS was employed, the migration times of DNA fragments decreased linearly by increasing the field from 117.6 to 152.9 V/cm, while the resolution quality increased. At the electric field of 141.2 V/cm, the 50-bp DNA fragments showed a short analysis time with baseline separation at the BS (Fig. 5B), while the DNA fragments at the conventional MCGE (Fig. 5E) did not. Within the low-conductivity zone of a high electric field, the DNA molecules move at higher velocities than in the untitrated $Tris^+$ buffer zone. The DNA fragments were concentrated at the edge of the neutralized zone, because of BS, and showed decreased migration times. Above the electric field of 152.9 V/cm, none of the DNA fragments were perfectly separated, due to the high electric field. However, at the optimum conditions of MCGE-BS (Figs. 4B and 5B), resolutions of different size DNA fragments were maintained.

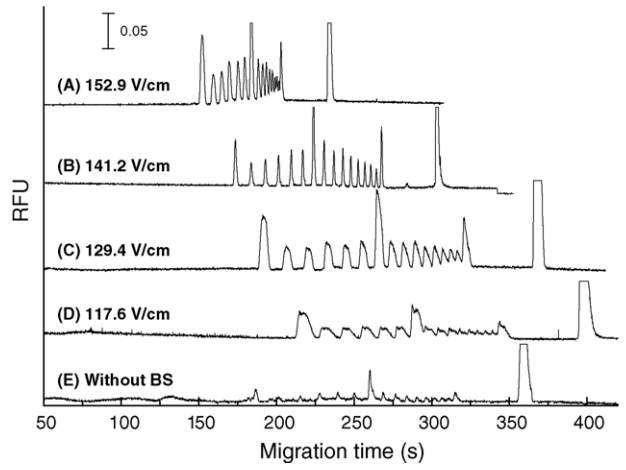


Fig. 5. Comparison of separation voltage for microchip gel electrophoresis with base stacking. Applied electric field conditions: (A) 152.9 V/cm, (B) 141.2 V/cm, (C) 129.4 V/cm, (D) 117.6 V/cm and (E) conventional electrokinetic injection without BS, 141.2 V/cm. Other MCGE-BS conditions are as shown in Fig. 4.

A comparison of MCGE separation with BS and without BS at the separation condition was shown in Fig. 6. At the MCGE-BS, all DNA fragments of the 50-bp DNA ladder were separated within 5.3 min without a significant loss in baseline resolution, and with high efficiency (Fig. 6A). The average calculated signals ($n = 5$), based on the peak height measurements, were enhanced 3.9–8.0-fold in the MCGE-BS separation, when compared to those of the normal MCGE (Table 1). In particular, the intensity of the 650-bp DNA fragment was enhanced 8.0-fold by BS just by using a hydroxide ion and a conventional double-T microchip in the given conditions (Table 1 and Fig. 6). When compared to conventional MCGE separation, the MCGE-BS method easily increased the detection sensitivity for the analysis of a 50-bp DNA ladder (50–2650-bp DNA fragments) by simply using 0.1 M NaOH.

3.2. Application of the MCGE-BS for a clinical sample

The MCGE separations, with and without BS, of the amplified 816-bp DNA fragment from the 18S rRNA of *T. buffeli* for the diagnosis of bovine *theileria* are compared in Fig. 7.

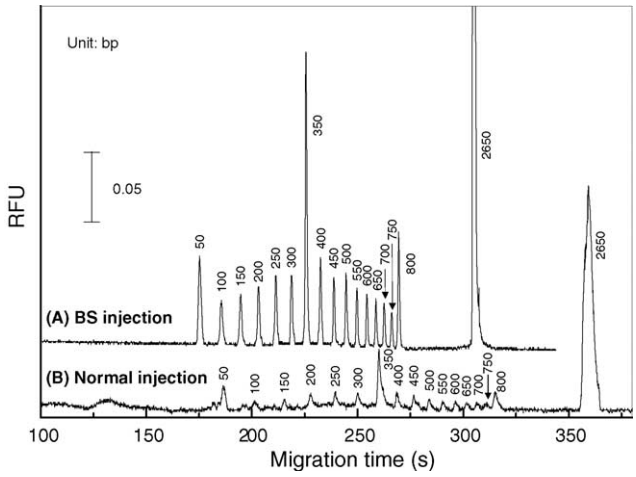


Fig. 6. Comparison of MCGE separation (A) with BS and (B) without BS at optimum condition. Other MCGE conditions as shown in Fig. 4.

Table 1
Comparison of the ratios of peak area and peak height of DNA fragments obtained by the conventional MCGE method without base stacking (A) and the MCGE-BS method (B)

DNA fragments (bp)	Peak height ratio A:B	Peak area ratio A:B
50	1:3.9	1:2.7
100	1:5.7	1:4.2
150	1:6.1	1:5.4
200	1:4.5	1:2.7
250	1:5.1	1:3.2
300	1:6.9	1:4.5
350	1:5.2	1:2.7
400	1:6.2	1:4.4
450	1:5.1	1:2.6
500	1:7.1	1:4.0
550	1:7.0	1:5.2
600	1:6.6	1:4.2
650	1:8.0	1:5.2
700	1:7.2	1:3.9
750	1:5.4	1:1.9
800	1:6.7	1:2.6
2650	1:5.1	1:1.2

According to previous studies [26,27], there was no notable difference between the PCR products of the purified DNA sample and the whole blood sample. The content of the amplified 816-bp DNA fragment in the PCR products of purified DNA was about four times higher than that of the whole blood sample. Sometimes, however, the poor detection sensitivity of DNA fragments for PCR assays from clinical samples can lead to an incorrect diagnosis. By using the MCGE-BS for the diagnosis of bovine *theileria*, the mean peak height ($n = 5$) was increased 7.0-fold, compared to that of normal MCGE without BS. There were no other peak-broadening effects or increasing migration time. This data indicates that the detection sensitivity of MCGE-BS can easily be achieved using an amplified PCR DNA fragment with conventional electrokinetic injection mode and base stacking. This process provides sufficient sensitivity for clinical analysis, such as the diagnosis of bovine *theileria*, without any chip modification.

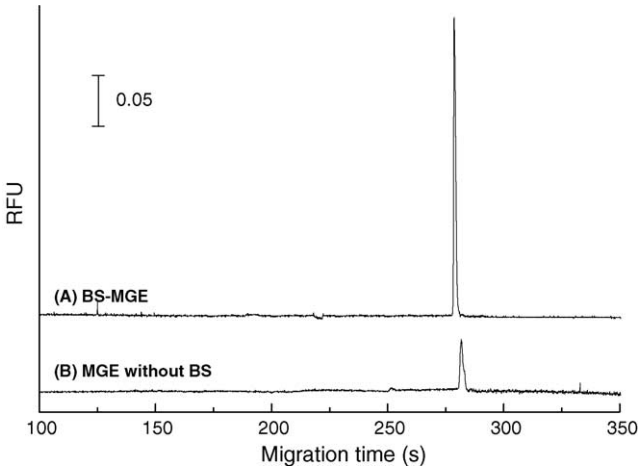


Fig. 7. MCGE separation (A) with base stacking, and (B) without BS (conventional electrokinetic injection) for the diagnosis of bovine *theileria* PCR product. (A) MCGE separation with the base stacking: OH^- (0.1 M NaOH) injection electric field, 533.3 V/cm for 20 s; DNA sample injection electric field, 600.0 V/cm for 60 s; separation electric field, 141.2 V/cm. (B) MGE separation without BS: DNA sample injection electric field, 600.0 V/cm for 60 s; separation electric field, 141.2 V/cm. Other MCGE conditions were the same as shown in Fig. 6.

4. Conclusions

Preparing the DNA sample in the same electrophoresis buffer, but at a lower conductivity, causes the sample resistance and field strength (V/cm) in the sample plug to increase [32]. In turn, the neutralization reaction between OH^- ions and $Tris^+$ ions causes the DNA molecules to migrate rapidly and stack as a sharp band at the edge of the low-conductivity zone (neutralized zone). As a result of the BS of DNA molecules, the DNA fragments were concentrated by up to 8.0-fold. The BS mechanism was very simple, and useful in the analysis of different size DNA fragments in MCGE-BS, as it provided enhanced sensitivity, high plate numbers, and better separation, without additional complex steps and/or chip modification. The BS in microchip was induced by simple electrokinetic injection of hydroxide ions. A neutralization reaction between these OH^- ions and the cationic buffer component $Tris^+$ resulted in a zone of lower conductivity, which increased the sample resistance and the field strength (V/cm) in the sample plug. The process caused a field focusing of different size DNA fragments in the microchip channel.

In addition, as a result of high field effects, excessive heat can be generated in the sample plug, causing band broadening and thermal degradation in some of the MCE components. Generally, the ratio of on-line sample stacking (or concentration) in MCE is proportional to the OH^- ions and DNA sample injection amount. The same phenomena should be observed in the MCGE-BS of the microchip. Consequently, if we want to significantly enhance the sensitivity, a modified microchip is needed to increase the OH^- ions and the DNA sample amount.

Acknowledgements

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